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In Vitro Transcription of Defective Interfering Particles of Influenza Virus Produces Polyadenylic Acid-Containing Complementary RNAs

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Influenza virus defective interfering (DI) RNAs, which originate from polymerase genes by simple internal deletion, can be transcribed in vitro. These DI RNA transcripts contain covalently linked polyadenylic acid, and their synthesis is dependent on ApG or capped RNAs as primers. Furthermore, like the standard viral RNA transcripts, they are complementary in nature and are slightly smaller in size compared with the corresponding DI RNAs. Hybridization of the specific DI RNA transcripts with the corresponding DI RNA segments and analysis of the duplex RNA by gel electrophoresis indicate that they are not incomplete polymerase gene transcripts, but rather the transcripts of the DI RNAs. Since influenza virus DI RNAs contain both the 5′ and the 3′ termini and transcribe polyadenylic acid-containing complementary RNAs in vitro the mechanism of interference may differ from that of the 5′ DI RNAs of Sendai and vesicular stomatitis viruses.

Repeated high-multiplicity passages of influenza virus yield defective interfering (DI) particles (14, 24, 31) which contain, in addition to other viral RNA segments, small RNA molecules (DI RNA) that are not present in standard viruses (26). These DI RNA molecules have been shown to be responsible for interference (16) and present in many stock virus preparations (15). All influenza DI RNAs studied to date have been shown to arise from the P1, P2, or P3 polymerase genes (7, 30). Furthermore, a single polymerase gene can give rise to DI RNAs of multiple sizes (7, 24, 30).

Based on the sequence relationship to the progenitor RNA, DI RNAs are either 5′ DI RNA or 5′-3′ DI RNA depending on whether the 5′ terminus or both the 5′ and the 3′ termini of the progenitor RNA are preserved in the DI RNA (21, 25). In addition, a class of mosaic virus (or compound) DI RNA that contains additional structural rearrangement has been described (18, 22, 23). Sequence studies have shown that the majority, if not all, of influenza virus DI RNAs belong to the 5′-3′ type (6, 25; Nayak and Sivasubramanian, unpublished data). On the other hand, the majority of vesicular stomatitis virus RNAs are of the 5′ type and transcribe a 46-mer (12), with the exception of DI LT which is of the 5′-3′ type and produces transcripts corresponding to the 3′ half of the genome (4).

The molecular mechanism of interference mediated by influenza virus DI remains unknown at the present time. Since both 5′ and 3′ genomic sequences are present in influenza virus DI RNAs, we postulated (25) from the sequence studies that 5′-3′ DI RNAs, unlike 5′ vesicular stomatitis virus DI RNAs, should be capable of undergoing transcription and possible translation in infected cells and therefore may interfere by a mechanism different from that postulated for the 5′ DI RNAs (13, 27). In this communication we demonstrate that, indeed, purified influenza virus DI particles behave similarly to the standard virus in their capacity to synthesize polyadenylic acid [poly(A)]-containing complementary RNAs (cRNAs) in vitro.

MATERIALS AND METHODS

Cells and virus. Procedures for culturing MDBK cells and growing A/WSN/33 (H1N1) as well as the DI viruses have been described previously (26). For the preparation of ^{32}P-labeled virus, carrier-free ^{32}P was present in the medium throughout the infection. In this study DI-L, DI ts^+ (Tob), DI 2-13, and standard ts^+ and *ts*52 viruses have been used (8, 15).

Purification of virus. The maintenance medium containing the virus was centrifuged at 3,000 rpm for 10 min at 4°C to remove the cellular debris, layered on a 15-ml discontinuous sucrose gradient (20 to 60% [wt/wt] in 0.1 M NaCl, 10 mM Tris-hydrochloride [pH 7.4], and 1 mM EDTA [NTE]) and centrifuged at 25,000 rpm in an SW27 Spinco rotor for 2 h at 4°C. The virus band was collected by puncturing the tube,

diluted four times with NTE buffer, layered on an 18-ml linear sucrose gradient (30 to 60% in NTE buffer), and centrifuged at 25,000 rpm for 2 h. Again, the virus band was collected, diluted with NTE, and pelleted by centrifugation for 2 h in an SW27 rotor at 25,000 rpm. The pellet was dissolved in NTE buffer (pH 7.4) without EDTA and used for transcription studies.

Polyacrylamide gel electrophoresis of viral RNA. Procedures for the extraction of ^{32}P-labeled as well as unlabeled viral RNA have been described (26). The RNA was analyzed on 2.2% polyacrylamide–0.6% agarose gels containing 6 M urea–36 mM Tris base–30 mM NaH_2PO_4–10 mM EDTA as described previously (10).

In vitro transcription. In vitro transcription was done by the method of Plotch and Krug (28). Purified DI particles were disrupted with Triton N-101 (0.2%) in 0.05 M Tris-hydrochloride (pH 8.0)–0.1 M KCl–0.01 M NaCl–5 mM $MgCl_2$–1 mM dithiothreitol–1 mM ATP–1 mM GTP–1 mM UTP–50 μM CTP (total volume, 0.2 ml) with 0.4 mM ApG or 12 μg of 2′-*O*-methyl-alfalfa mosaic virus RNA 4 as primers. [α-^{32}P]CTP was used as the labeled triphosphate. The mixture was incubated at 31°C for 2 h. The amount of radioactivity incorporated was determined by trichloroacetic acid precipitation and was counted in a liquid scintillation counter with a toluene-based scintillation fluid.

Isolation of the RNA product. After incubation for 2 h at 31°C, the reaction mixtures were made 0.5% in sodium dodecyl sulfate, 0.001 M in EDTA, and 0.1 M in Tris-hydrochloride (pH 9.0), and the RNA was deproteinized by extracting three times with phenol-chloroform (1:1) at pH 9.0 and finally another extraction with chloroform alone.

Analysis of the RNA product. (i) Oligo(dT)-cellulose chromatography. RNA was dissolved in 0.5 M LiCl–0.01 M Tris-hydrochloride (pH 7.5)–0.5% lithium dodecyl sulfate and was applied to a column of oligodeoxythymidylic acid [oligo(dT)]-cellulose equilibrated with the same buffer (28). The eluate was reapplied to the column, and the column was washed with the buffer until all unbound radioactivity had been removed. The bound RNA was then eluted with 0.01 M Tris-hydrochloride (pH 7.5), and the RNA in both fractions was recovered by precipitation with ethanol.

(ii) Polyacrylamide gel electrophoresis. The in vitro product RNA was analyzed by polyacrylamide agarose gel electrophoresis as described above. Analysis of the double-stranded RNA was done as described previously (11).

(iii) RNase sensitivity of product RNA. The product RNA in 0.01 M Tris-hydrochloride (pH 7.5)–0.002 M EDTA was heated for 5 min at 100°C and fast cooled. The salt concentration was increased to 2.5 × SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate), the RNA was digested with a combination of RNases A (4 μg/ml) and T_1 (20 U/ml) for 1 h at 37°C, and the acid-insoluble radioactivity was determined (1, 5).

(iv) RNA-RNA hybridization. The product RNA was mixed with an excess of viral RNA and heated for 5 min at 100°C. The salt concentration was increased to 2.5× SSC. RNA was hybridized at 80°C for 4 h and cooled slowly to room temperature. The percentage of the product which became RNase (A and T_1) resistant was determined.

Size of the poly(A) sequences. To determine the approximate length of the poly(A) sequences in the product RNA, in vitro [α-^{32}P]AMP-labeled product RNA was mixed with yeast RNA (100 μg/ml), heated, fast cooled, and digested with both RNases A and T_1 as described above. The RNase-resistant fragments were subjected to electrophoresis on a 15% polyacrylamide gel containing 6 M urea at 1,000 V for 18 h. ^{32}P-labeled yeast tRNA (80 nucleotides) and [^{32}P]polydeoxythymydylic acid (400 nucleotides) were used as molecular weight markers.

Synthesis of [^{32}P]ApG. ApG (1 mM) was incubated for 2 h at 37°C with 10 U of polynucleotide kinase in a final volume of 30 μl containing 50 mM Tris-hydrochloride (pH 8.0), 4 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM spermidine-hydrochloride, and 1 mCi of [γ^{32}P]ATP. The reaction mixture was then electrophoresed on a 5% polyacrylamide gel for 5 h at 1,000 V. The band of ^{32}P-labeled ApG was electroeluted from the gel, and 20 to 30% was recovered by ethanol precipitation.

Determination of site(s) of incorporation of [^{32}P]ApG in the in vitro product RNA. A transcription mixture containing 0.2 mM [^{32}P]ApG in a total volume of 0.2 ml was incubated for 3 h at 31°C. Subsequently, the RNA was isolated by phenol-chloroform extraction and selected for poly(A) by oligo(dT)-cellulose chromatography. Poly(A) was then removed by hybridization to polydeoxythymidylic acid and RNase H treatment (9). The deadenylated transcript was subjected to gel electrophoresis as described above. The DI RNA transcripts from the corresponding bands in the gel were electroeluted, precipitated with ethanol, and digested with 30 U of RNase T_2 per ml at 37°C for 5 h in 0.03 M sodium acetate (pH 4.5). After phenol-chloroform extraction the hydrolysate was fractionated with a DEAE-52 column in 7 M urea. The material eluted from the column was further analyzed by 3MM paper electrophoresis as described previously (1).

RESULTS

Kinetics of in vitro transcription. The transcription in vitro was linear at least up to 1 h at 31°C for both DI particles and standard viruses (Fig. 1), although the reaction for standard virus often continued linearly up to 3 h (Fig. 1). Transcriptase activities in the linear phase of the reaction (i.e., up to 1 h) with ApG as the primer were as follows: WSN *ts*$^+$ (3,723 pmol/mg per h), DI *ts*$^+$ (Tob) (3,000 pmol/mg per h), DI L (2,112 pmol/mg per h), and DI 2-13 (792 pmol/mg per h). The transcription of both standard virus and DI particles was stimulated 100-fold by ApG as well as by 2′-*O*-methyl capped alfalfa mosaic virus RNA 4 as primers, but not by UpG. Essentially similar results were obtained by Plotch and Krug (28) with standard virus.

Nature of the DI RNA transcripts. Since DI L RNAs have been characterized extensively by oligonucleotide mapping (8) and RNA sequencing (9) and since the complete sequences of three DI L RNAs (L2a, b, and L3) are known (25; Nayak, unpublished data), detailed analyses of the nature of the transcripts were done with DI L particles. Figure 2 shows the migration pattern

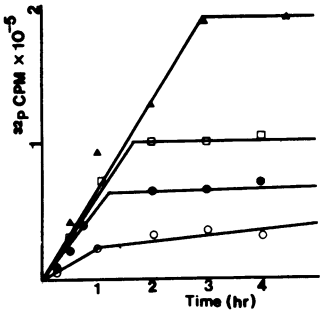

FIG. 1. Kinetics of in vitro transcription. Transcription was done using 24.7 μg of DI L (●), 21.3 μg of DI WSN *ts*$^+$ (Tob) (□), and 30.3 μg of DI 2-13 (○) as well as 18.2 μg of standard WSN virus (▲) in an in vitro transcription system (0.2 ml volume) as described in the text. At different times 20-μl samples were taken out from the reaction tubes and precipitated with 5% trichloroacetic acid containing 0.05 M sodium pyrophosphate. The filters were dried and then counted in a liquid scintillation counter with a toluene-based scintillant.

of the RNA synthesized by DI particles and standard viruses in the presence of either ApG or capped alfalfa mosaic virus RNA as a primer. Poly(A) sequences in viral transcripts were hybridized to oligo(dT) and subsequently removed by digestion with RNase H isolated from calf thymus (9). The DI particles synthesized cRNA segments from both the genomic RNA and DI RNA segments. These newly synthesized transcripts moved slightly faster than the corresponding uniformly ^{32}P labeled viral or DI RNA segments, indicating that the in vitro transcripts are shorter than the corresponding viral (9) or DI RNA segments. Also, as expected, the transcripts primed by capped RNAs (Fig. 2A, lane 3) were slightly larger than those primed by ApG (Fig. 2A, lane 2). Similarly, two other DI preparations [DI *ts*$^+$ (Tob) and DI 2-13] produced transcripts which corresponded to the DI RNA segments present in these DI particles, but different from that present in DI L particles (data not shown). Furthermore, as expected, the standard virus preparation produced only the transcripts which corresponded to the genomic RNA segments (Fig. 2B), and the DI RNA specific transcripts were absent.

To determine the variation of transcription among the different genomic and DI RNA segments the molar ratios of the genomic RNA segments and the corresponding transcripts relative to the segment M were determined from the counts present in each band (Table 1). The molar ratio of the larger vRNA segments, even in the standard virus preparation, was often reduced, and the transcription of polymerase genes was even further reduced as has been observed for both in vivo and in vitro transcripts (28). However, there was no marked differential synthesis of cRNAs among the DI RNA segments even when the concentration of DI particles was increased in the reaction mixture (Fig. 2, lanes 2, 4, and 5). To determine whether the transcripts were full-length copies of DI RNAs, specific RNA segments were eluted from gels and hybridized to the corresponding minus-strand DI RNA also eluted from gels. Hybrids were treated with RNases A and T_1 and analyzed in gels

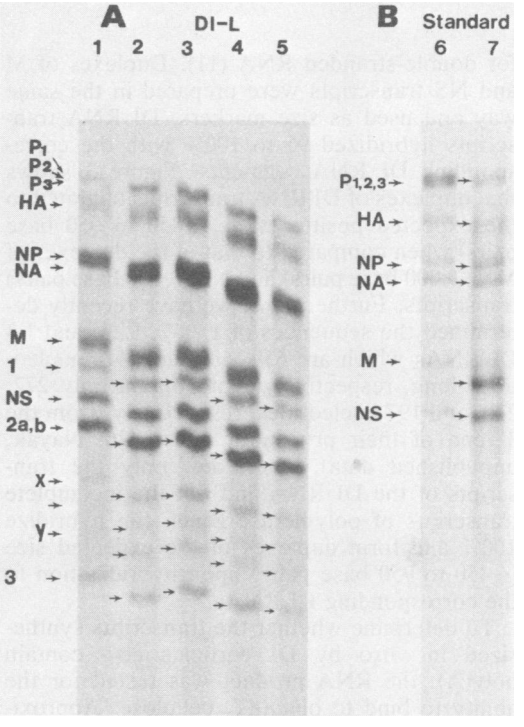

FIG. 2. Polyacrylamide gel electrophoresis of in vitro transcription product. RNA syntheses were performed with Triton N-101-disrupted viruses in a reaction volume of 0.2 ml with either ApG or capped 2′-*O*-methyl alfalfa mosaic virus RNA 4 as primers as described in the text. The mixture was incubated for 2 h at 31°C. The RNA products were isolated, deadenylated, and then subjected to electrophoresis on 2.2% polyacrylamide and 0.6% agarose gel for 17 h at 140 V at 25°C. (A) RNA transcription products of DI L. Lanes 1, 2, and 3 represent ^{32}P-labeled genome RNA and ApG-primed and alfalfa mosaic virus-primed transcription products, respectively. Lanes 4 and 5 are the same as lane 2, but at different virus concentrations (300 and 500 μg/ml respectively). In lane 2 150 μg of virus per ml was used. Numbers 1, 2a, b, 3, x, and y in lane 1 show the position of DI RNAs. Arrows in lanes 2 through 5 show the corresponding DI RNA transcripts. (B) The standard virus genome (lane 6) and transcripts of standard virus in the presence of ApG as the primer (lane 7).

TABLE 1. Molar ratios of transcripts of specific viral and DI RNA segments[a]

RNA		Molar ratio							
		Viral segments					DI segments		
		P1 + P2 + P3	HA	NP + NA	M	NS	1	2a + 2b	3
DI L	viral RNA	0.09	0.44	0.60	1.0	1.18	0.55	0.69	1.28
DI L	cRNA	0.07	0.39	0.52	1.0	1.14	0.48	0.62	1.14
Standard	viral RNA	0.29	0.62	0.58	1.0	1.10			
Standard	cRNA	0.10	0.59	0.45	1.0	1.06			

[a] RNA transcription was carried out as described in the text. The labeled viral RNA and cRNA were analyzed by polyacrylamide-agarose gel electrophoresis as described in the legend to Fig. 2. The molar ratio of each band relative to M (either viral RNA or cRNA) was determined by dividing the counts present in the specific band by the nucleotide number of that RNA.

for double-stranded RNA (11). Duplexes of M and NS transcripts were prepared in the same way and used as size markers. DI RNA transcripts hybridized 95 to 100% with the corresponding DI RNA segments. Figure 3 shows that duplexes of DI RNA transcripts migrated to the expected position (i.e., ~450 to 950 base pairs) when compared to that of the duplexes of M (~1,000 base pairs) and NS (~870 base pairs) transcripts. Furthermore, we have recently determined the sequences of L2a-7, L2b, and L3 DI RNAs which are 659, 683, and 441 nucleotides long, respectively, but contain only 272, 270, and 197 nucleotides, respectively, from the 3′ end of their progenitor genes (26; Nayak, unpublished data). Therefore, only the transcripts of the DI RNA and not the incomplete transcripts of polymerase genes can hybridize 100% and form duplexes of the expected size (~450 to 950 base pairs) upon hybridization to the corresponding DI RNAs.

To determine whether the transcripts synthesized in vitro by DI particles also contain poly(A), the RNA product was tested for the ability to bind to oligo(dT) cellulose. Approximately 60 to 75% of the in vitro DI cRNA bound to oligo(dT)-cellulose, as has been reported for the transcripts of standard virus (9). After removal of poly(A) by polydeoxythymidylic acid hybridization and RNase H treatment, the RNA product was no longer capable of binding to oligo(dT)-cellulose. Another indication that the product RNA contains poly(A) sequences came from the observation that [^{32}P]CMP- and [^{32}P]AMP-labeled RNA transcripts were differentially sensitive to treatment with RNases A and T_1. Transcripts were denatured by heat and rapid chilling to eliminate secondary structure before digestion with A and T_1 RNases. The RNase resistance to [^{32}P]CMP-labeled transcripts was markedly different. Only 2 to 4% of the [^{32}P]CMP-labeled transcripts was RNase resistant, whereas 24 to 27% of the [^{32}P]AMP-labeled transcript was resistant to RNases. Both of the labeled transcripts became 95 to 100% resistant to RNase after hybridization to the genomic RNA at a concentration of 2 μg/ml. Finally, to determine the approximate length of poly(A) sequences in the transcripts, [^{32}P]AMP-labeled product RNA was mixed with tRNA, heated, rapidly cooled, treated with RNases, and then subjected to electrophoresis on 15% polyacrylamide gels containing 6 M urea as described above along with appropriate RNA markers (5). The size of poly(A) is approximately 60 to 350 nucleotides in length (data not

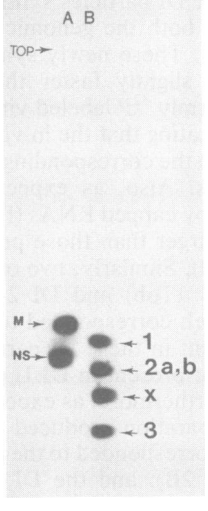

FIG. 3. Analysis of the DI RNA duplexes. DI L-specific transcripts (Fig. 2A) were electroeluted from the gel and hybridized with the corresponding unlabeled DI RNA segments which had similarly been isolated from gels with ^{32}P-labeled RNA as a marker in the next lane. The hybrids were then mixed and treated with RNases (A and T_1), and the double-stranded RNAs were separated by electrophoresis on a 4% polyacrylamide gel at 90 V for 18 h (lane B). As a control duplexes of NS and M were run side by side (lane A).

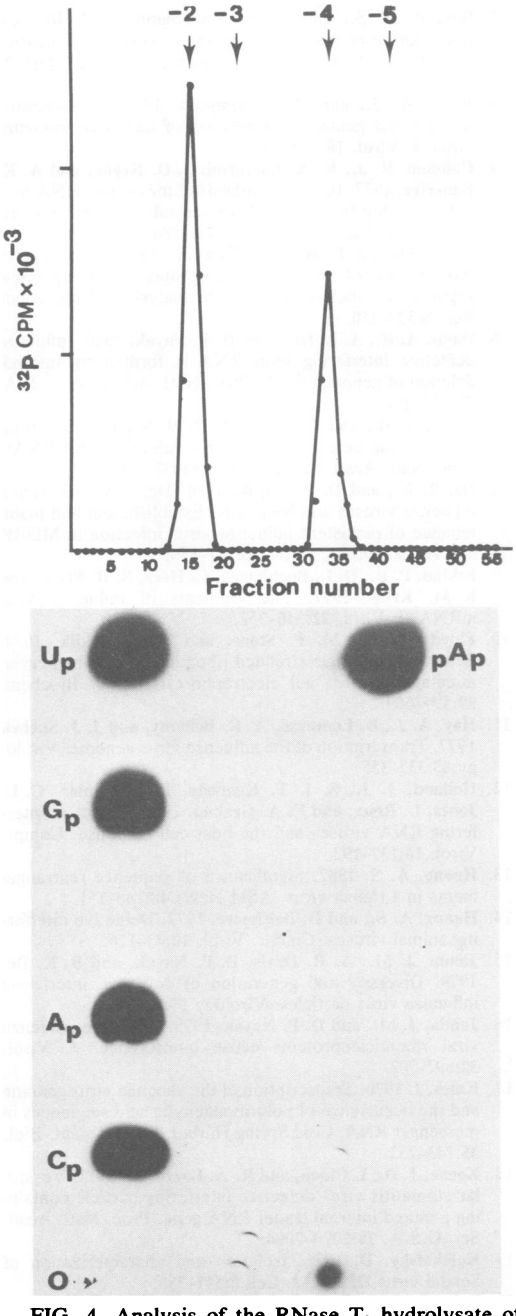

FIG. 4. Analysis of the RNase T_2 hydrolysate of the ^{32}P-labeled ApG-stimulated DI RNA transcripts. Transcription products of DI L were isolated by gel electrophoresis (Fig. 2A). Specific DI RNA bands were electroeluted from gel segments, ethanol precipitated, and digested to completion with RNase T_2. (A) the hydrolysate was then chromatographed on a DEAE-52 column in 7 M urea. Arrows indicate the position of ^{3}H markers for charges. (B) The materials at charge −2 and −4 were analyzed by 3MM paper electrophoresis. Unlabeled 5′ AMP, 5′ CMP, 5′ GMP, 5′ UMP, and pAp were added as UV markers to identify their positions.

shown) and, as reported, is more heterogeneous when compared with the poly(A) synthesized in vivo (17, 28).

Demonstration that ApG acts as an initiator of the RNA synthesized in vitro. To demonstrate that ApG acts as an initiator of RNA transcription, ApG labeled at its 5′ end with polynucleotide kinase and [γ-^{32}P]ATP was used in the RNA transcription. The transcription products were separated by polyacrylamide agarose gels after removing poly(A) sequences as described above. The transcription products corresponding to the DI RNAs were eluted from gels and digested completely with T2 RNase. The hydrolysate was then analyzed by chromatography on a DEAE-52 column in 7 M urea. Two peaks of ^{32}P were found, one at the charge of −2 and the other at the charge of −4 (Fig. 4A). Analysis of the −2 peak by 3MM paper electrophoresis demonstrated the presence of all four labeled mononucleotides (Fig. 4B). This clearly shows that ^{32}P had been internally incorporated. Similar internal incorporation from the formation of [α-^{32}P]ATP by the virion-associated nuclease and phosphokinase has been described previously (28). The material at the −4 peak, when analyzed by 3MM paper electrophoresis, comigrated with the authentic pAp. This shows that ^{32}P-labeled ApG was really incorporated into the 5′ end of the mRNA and therefore served as a primer for the RNA transcription.

DISCUSSION

In the present study we have shown that the DI particles are capable of synthesizing in vitro cRNAs which possess characteristics similar to those of standard RNA transcripts (9, 20). The conditions for transcription were essentially the same as reported by Krug and his colleagues (9, 28), i.e., it required the presence of Mg^{2+} and a dinucleotide primer (ApG) or capped RNA. In earlier studies Bean and Simpson (3) also reported that defective influenza viruses also contain polymerase activity and transcribe eight standard RNA segments. However, our report clearly shows that not only are the standard RNA segments in DI particles transcribed, but the DI RNA segments are also transcribed into poly(A)-containing cRNA, indicating that influenza virus DI RNAs can also function as a template for transcription.

Experimental data indicate that the transcripts found in DI preparations are the copies of the DI RNAs and not incomplete transcripts of polymerase genes. (i) The size and the number of transcripts clearly correspond to the size and number of DI RNA segments in a given DI preparation (Fig. 2A). Transcripts corresponding to the specific DI RNAs were also found in two other DI preparations [DI ts^+ (Tob) and DI

2-13) that have been examined (data not shown). The sizes of the DI transcripts vary in different DI preparations and correspond to the size of the DI RNAs present in a given DI preparation. (ii) Furthermore, these specific transcripts are absent in the product of standard virus (Fig. 2B). (iii) Since these in vitro transcripts contain ApG as a primer at the 5′ end and poly(A) at the 3′ end and since poly(A) addition sites are presumed to be at the proximity of the 3′ end of standard RNA transcripts (29), these DI-specific transcripts are unlikely to be incomplete transcripts of polymerase genes. (iv) Finally, since the complete sequences of a number of DI RNAs used here are known and since they are internally deleted containing only a portion (40 to 60%) of sequences from either the 3′ or the 5′ end of the progenitor gene (25), incomplete transcripts of P genes cannot hybridize 100% with DI RNAs. Duplexes of expected size (~450 to 950 base pairs) can be formed only with the transcripts of the corresponding DI RNA and not with the incomplete transcripts of the progenitor genes.

The mechanism of interference by influenza DI RNAs remains unclear. Since, unlike 5′ DI RNAs, the 5′-3′ DI RNAs can be transcribed, they may interfere at the level of primary transcription of standard genome. Indeed, such a mode of interference has been suggested for vesicular stomatitis virus DI LT which is also of the 5′-3′ type (2). Alternatively, since the DI transcripts possess the characteristics of mRNAs, they may be translated and produce defective proteins which may, in turn, bind preferentially to the specific region(s) of genomic template(s) and inhibit the transcription or the replication (or both) of the standard RNA(s). Finally, the transcription of DI RNAs may have no functional role in interference, but other considerations such as the structure or the size of DI RNAs may be of significance in interference as has been postulated for 5′ DI RNAs (20). Detailed in vivo studies of both the transcription of the standard and DI RNA segments and the translation of genomic and DI transcripts would elucidate the mechanism of interference by 5′-3′ DI influenza viruses.

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